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Original Article

DEVELOPMENT AND VALIDATION OF A 96- MICROWELL BASED ASSAY OF AZITHROMYCIN BY CHARGE TRANSFER COMPLEXATION WITH 2, 3-DICHLORO-5, 6-DICYANO-1, 4- BENZOQUINONE (DDQ)

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ABSTRACT

A novel 96-microwell-based visible spectrophotometric assay was developed and validated for the determination of Azithromycin in tablets and capsules. The formation of a coloured charge-transfer complex between Azithromycin as n-electron donor, and 2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone, as π-electron acceptor, was investigated and employed as a basis for the development of the proposed assay in a 96 micro well Thermomax plate reader. A UV-Visible spectrophotometer was used for the study of the complex behaviour at 518 nm, while Thermomax 96-microwell plate reader was used for the content determination of Azithromycin tablet and capsule brands at 450 nm. The optimum conditions of the reaction and the analytical procedures of the assay were established. Under the optimum conditions, linear relationship with good correlation coefficient was found between the absorbance and the concentration of Azithromycin in the range of 0.026 – 0.105 mg/ml, with the equation y=0.048x and R^2= 0.984. The Gibbs free energy, ΔG, was found to be negative at different temperatures. The limits of detection and quantisation were found to be 0.023 and 0.069 mg/ml respectively. The procedure gave good precision. The assay was applied successfully to the analysis of azithromycin in tablets and capsules with satisfactory result. The assay described has high throughput property, consumes little volume of organic solvent, offers reduction in analysis cost and the exposure of the analyst to the toxic effects of organic solvent.

KEYWORDS: Charge-Transfer Complexation, DDQ, Azithromycin, Spectrophotometric Determination, Thermodynamic Studies

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INTRODUCTION

Macrolides are class of compounds used in human and veterinary medicine as antibiotics. They interfere in the synthesis of bacterial proteins resulting in a bacteriostatic effect on pathogens (*Mazzei, T.; Mini, E.; Novelli, A.; Periti, p.; 1993*).

Macrolides contain a macrocyclic lactone and a neutral sugar moiety attached to the lactone. It also contain other sugar moiety containing a dimethylamine group, which confers to the macrolides a basic behaviour (*Korolkovas, A.; 2006*) and makes them potential n-electron donating substances.

One of the most important members of this class is the azithromycin, which has been used as antibiotic in the human medicine for the treatment or prophylaxis of a number of health problems such as ear, lung, skin, and throat infections (*Guven, M.; Bulut, Y.; Sezer, T.; Aladag, I.; Eyibilen A.; Etikan, I.;2006, Peters, D. H.; Friedel, H.*

A.; MacTavish, D.; 1992), veneraldisease(*Drew, R. H.; Gallis, H. A.; 1992*) pneumonia(*Kinasewitz, G.; Wood, R. G.;1991*) toxoplasmosis(*Chang, H. R.; 1996*) and opportunistic diseases in patients with AIDS(*McCutchan, J. A.; 1996*)

Some analytical methods for azithromycin are published in literature. High performance liquid chromatography (HPLC) with different detectors is the most employed technique for this purpose. Hence, methods based on HPLC with electrochemical(*Leal, C.; Codony, R.; Compañó, R.; Granados, M.; Dolors-Prat, M.; 2004, Kees, F.; Spangler, S.; Wellenhofer, M.; 1998*) fluorimetric (*Wilms, E.; Trumpie, H.; Veenendaal, W.; Touw, D.;, 2005, Bahrami, G.; Mohammadia, B.; 2006*)and mass spectrometric (*Nirogi, R. V. S.; Kandikere, V. N.; Shukla, M.; Mudigonda, K.; Maurya, S.; Boosi, R.; Yerramilli, A.; 2005*)detection were already reported. On the other hand, the development of HPLC methods with UV detection is not a common subject because of the extremely low absorption of azithromycin. HPLC methods are usually costly, consumes much reagent and technically difficult in many developing countries. Zubata *et al.*(*2002*), proposed a simple and low-sensitivity methodology for the azithromycin determination in pharmaceuticals using this approach.

Other analytical techniques also employed for azithromycin determination were voltammetry (*Nigovic, B.; Imunic, B.S.; 2003*), amperometry (*Palomeque, M. E.; Ortíz, P. I.; 2007*), spectrofluorimetry (*Khashaba, P. Y.; 2002*), chemiluminescence (*Song, Z.; Wang, C.; 2003*) and spectrophotometry (*Rachidi, M.; Elharti, J.; Digua, K.; Cherrah, Y.; Boukloze, A.; 2006*).

Sultana *et al. (2006)* studied the degradation of the azithromycin in strong acidic and alkaline media. The authors converted the azithromycin in a species with higher absorbing capacity, making possible to perform its determination in the samples. Maximum absorption of the product was at 482 nm when a concentrated solution of H_2SO_4 was used, while maximum absorption at 215 nm was for the sample obtained in basic medium (0.2 mol L^{-1} NaOH). In both cases, the samples were heated at 60 ºC for 30 min to achieve quantitative conversion of the azithromycin. This analytical procedure is laborious and time-consuming.

The method developed by Walash *et al.* (2007) was based on the formation of binary complexes between the macrolide antibiotics and eosin Y in aqueous buffered medium at pH 3.0. At this condition, binary complexes presented strong absorption in the range of 542-544 nm. The procedure was applied in the determination of azithromycin, erythromycin, roxithromycin and clarithromycin in commercial medicines

A charge-transfer (CT) reaction can be defined as a process in which two separate species come together and one or more electrons are transferred from one species to the other. It is possible to deploy charge transfer method in the assay of azithromycin, because it has a charge to donate to an acceptor molecule.

This study was aimed at investigating the optical and thermodynamic parameters of azithromycin-DDQ complexation as well as development of a UV spectrophotometer assay for azithromycin tablets based on the complication reaction.

Charge-transfer reactions have been used to impact colour to uv active molecules, making them amenable to spectro photometric determination in the visible region. The aim of this work was to investigate the CT complications parameters AZT-DDQ complex, to investigate thermodynamic parameters of the CT complex of AZT-DDQ, to develop and validate a 96-microwell assay for Azithromycin tablet based on the CT complications of AZT with DDQ and to employ the proposed method for the assay of some commercially available AZT tablets and capsules circulating in Awka, Anambra

state, Nigeria

EXPERIMENTAL

Materials and Methods

Azithromycin and DDQ were obtained from Juhel Nigeria Ltd, Enugu State, Nigeria and Merck, Germany respectively. Four brands of azithromycin tablets and two brands of azithromycin capsules, all commercially available in Nigeria, but of Indian origin, were obtained from Pharmacy shops in Awka, Anambra state, Nigeria. Tablets and capsules were stored in cool dry place prior to the experiments and assays were done before their expiry dates. Analytical grade of absolute methanol (SIGMA-ALDRICH, USA) was used.

All absorption measurements were made using the double-beam ultraviolet/visible Genway 6505 spectrophotometer with quartz cuvette and micro well-plate absorbance reader (THERMOMAX 5891, England). Ohaus analytical balance (PA214), 10 ml pipette, beakers, glass funnels, 96-microwell plate, multi-channel micropipette, test tubes, 100 ml volumetric flask was also used in the experiments.

Preparation of Stock Solutions of Azithromycin and DDQ

A 0.001 M solution of azithromycin in 100 ml was prepared by weighing 0.0749 g of azithromycin API. The azithromycin was dissolved in small volume of methanol in a volumetric flask. The solution was made up to 100 ml mark in a volumetric flask. A 0.001 M solution of DDQ in 100 ml was prepared by weighing 0.0227 g of DDQ. The weighed DDQ was dissolved in small volume of methanol in a volumetric flask. The solution was made up to 100 ml in a volumetric flask.

Determination of Wavelength of Maximum Absorption

Azithromycin in methanol solution was scanned using UV/Visible spectrophotometer Genway 6505, between 200 and 400 nm wavelengths. DDQ in methanol as well as Azithromycin – DDQ mixture were each scanned using UV/Visible spectrophotometer Genway 6505. The maximum absorption wavelength of DDQ solution and the complex were also determined in the Thermomax microwell reader.

Effect of Time on the Formation of Azithromycin-DDQ Complex

The absorbance of a mixture of 2 mL of 0.001 M azithromycin solution in methanol and 2 mL of 0.001M DDQ solution in methanol was determined at various time intervals at λ_{max}determined earlier, using the UV/Visible spectrophotometer, at room temperature against methanol blank and the reagent blank at intervals of 5 or 10 mins for a total of 170 minutes

Determination of Stoichiometry of the Complex Formation between Azithromycin and DDQ

Standard solutions (0.001 M) of each of azithromycin and DDQ were prepared. Serial dilutions of the standard solution were mixed according to the following ratios (0:10, 1:9... 9:1, 10:0). The mixtures were allowed to stand for 100 minutes and the absorbance value determined at 518 nm wavelengths using the UV/Visible spectrophotometer, against the blank of methanol and the DDQ. A graph of absorbance against VA/(VA+VD) was plotted. The mole ratio was then determined from the intersection of the curves.

In Silico Determination of Complexation Mode between Azithromycin and DDQ

The complexation mode between azithromycin and DDQ was determined by molecular docking simulation using Auto Dock Vina 4.0 (Trott*et al.,* 2010). The mol2 files of azithromycin and DDQ were extracted from ZINC database (Irwin *et al.*, 2012) and converted to PDBQT files using Auto Dock Tool (Morris, G. M. *et al., 2009*). The molecular docking simulation was carried out using Auto Dock Vina (Trott*et al.,* 2010) on a Linux platform with the following grid box parameters; Centre X = 0.696, Y = -2.286, Z = 0.277 and Size X = 18, Y = 20, Z = 16 . Results obtained were visualized in PyMol (The PyMol Molecular Graphic System, Version 1.8, LLC.)

Determination of Effect of pH on the AZT-DDQ complex Formation Study

A 0.001 M solution of azithromycin was mixed with a 0.001 M solution of DDQ, in the ratio of 1:2. Buffer solution (pH, 1.0) was added to make up the volume to 10 mL. The same treatment was done with buffers (pH, 2– 13) in different test tubes for colour development. These were kept for 100 mins to enable full development of the complex. The absorbance of the resulting complexes was determined at 518 nm wavelength using the UV/Visible spectrophotometer. A graphical plot of the pH against absorbance was done.

Determination of Association Constant, Molar Absorptivity and Thermodynamic Parameters of the AZT-DDQ Complex

Serial volumes (0.4, 0.8, ⋯, 1.8 mL) of azithromycin solution (0.001 M) were transferred into different test tubes. Serial volumes (0.8, 1.6 ⋯, 3.2 mL) of DDQ solution in methanol (0.001 M) were added to the various test tubes. The solutions were diluted to 6 mL with methanol mixed and left at room temperature for 100 minutes. Their absorbance values were determined at 518 nm wavelength at temperature of 25°C (room temperature). Further analysis of the reaction mixtures were done by subjecting them to temperatures of 40, 50, 60 and 70° C respectively in a thermostated water bath.

Azithromycin-DDQ complex*:* They were evaluated using a modification of the Benesi-Hildebrand Equation (*Benesi and Hildebrand, 1949*)

$$\frac{[D_O][A_O]}{A_\lambda^{[D:A_2]}} = \frac{1}{E_\lambda^{[D:A_2]}} + \frac{1}{K_C^{(D:A_2)} E_\lambda^{(D:A_2)}}\left(\frac{1}{[D_O]}\right)$$

Where $[D_o]$ and $[A_o]$ are the initial concentration of the reactants $A_\lambda^{(D:A)}$ is the absorbance of the complex at 490 nm, $E_\lambda^{(DA)}$ is the molar absorptivity of the complex at 490 nm, $K_C^{(DA)}$ is the stability constant. The plot of $[D_o][A_o]/ A_\lambda^{(DA)}$ against $[D_o]$ was made. The intercepts and slopes of the regression lines were used to obtain the values of $E_\lambda^{(DA)}$ and $K_C^{(DA)}$ respectively, at constant $[A_o]$.

Determination of Beer's Limit for the AZT-DDQ Complex

Different concentrations of azithromycin solution (0.001M) in DDQ (0.001M) were prepared and made up to 20 ml. The solutions were left for 100 minutes for maximum complexation. The absorbance values of the samples were taken in triplicate using GENWAY UV-Visible spectrophotometer at 518 nm, and their average value calculated. A Graphic plot of absorbance against concentration revealed the point beyond which Beer's law became inapplicable.

Identity Profile and Uniformity of Weight Test for Azithromycin Tablet and Capsule Formulations

Twenty tablets from each of the brands were individually weighed, and three readings were taken. The average of the three readings and the standard deviation were determined.

Assay of Azithromycin Tablet and Capsule Formulations

Amount equivalent to 0.001 M of azithromycin API was prepared from each of the brands in 50 ml of methanol. The solutions were filtered to remove the excipients. From each brand, 1 ml each of the filtrates were placed into a test tube and 2 ml of 0.001 M solution of DDQ solution added to each. The mixtures were made up to 10 ml by addition of 7 ml of methanol to each of the test tubes and left for 100 minutes for full complexation to take place. The absorbance of each was determined in the Thermomax microwell plate reader and their concentrations calculated from the Beers' plot of the AZT-DDQ Complex.

Validation Experiments for the Proposed Method

A 0.001 M of azithromycin solution and 0.001 M solution of DDQ were prepared. Different concentrations of azithromycin in DDQ were prepared and made up to 20 ml. The solutions were left for 100 minutes for maximum complexation. The absorbance values of the solutions were taken with the Thermomax microwell reader and there averages calculated.

The limit of detection (LOD) and limit of quantitation (LOQ) were estimated by obtaining three calibration curves for serial dilutions of azithromycin. The mean standard deviation and mean slope of the three graphical plots were determined. While the LOD was calculated using the formula, 3.3 x Standard deviation/slope, the LOQ was determined using the formula, 10 x Standard deviation/slope.

The inter-day precision of the method (repeatability) was evaluated by analyzing the absorbance values of a freshly prepared 0.12 mg % concentration of azithromycin daily for five days. Duplicate absorbance readings of the samples were obtained using the Thermomax microwell reader at 450 nm and investigated using percentage relative standard deviation.

The intra-day precision of the method was evaluated by analyzing 0.12 mg % of azithromycin. Duplicate absorbance readings of ten different aliquots were taken using the Thermomax microwell reader at 450 nm and investigated using percentage relative standard deviation.

RESULTS AND DISCUSSIONS

Results of Determination of Wavelength of Maximum Absorption

The graphical plot of absorbance versus wavelength for AZT, DDQ and AZT-DDQ complex is presented in figure1A**.** While the solution of DDQ in methanol absorbed maximally at 450 nm wavelength, the solution of AZT absorbed maximally at 216 nm wavelength. The mixture of AZT in methanol and DDQ in methanol absorbed maximally at 518 nm wavelength. From figure 1A**,** one can see that there was a bathochromic shift in the spectral band position of azithromycin (216 nm) to a much longer wavelength (518 nm) of the AZT-DDQ complex, because the red colour in the visible spectrum has a longer wavelength than most other colours, this effect is also commonly called a red shift. In the Thermomax microwell plate reader the maximum wavelength of absorption of the complex was 450 nm as shown in figure 1B. The difference in the maximum absorptions of the complex in both machines could be filter or machine dependent.

Thermomax device makes use of colorimetric techniques.

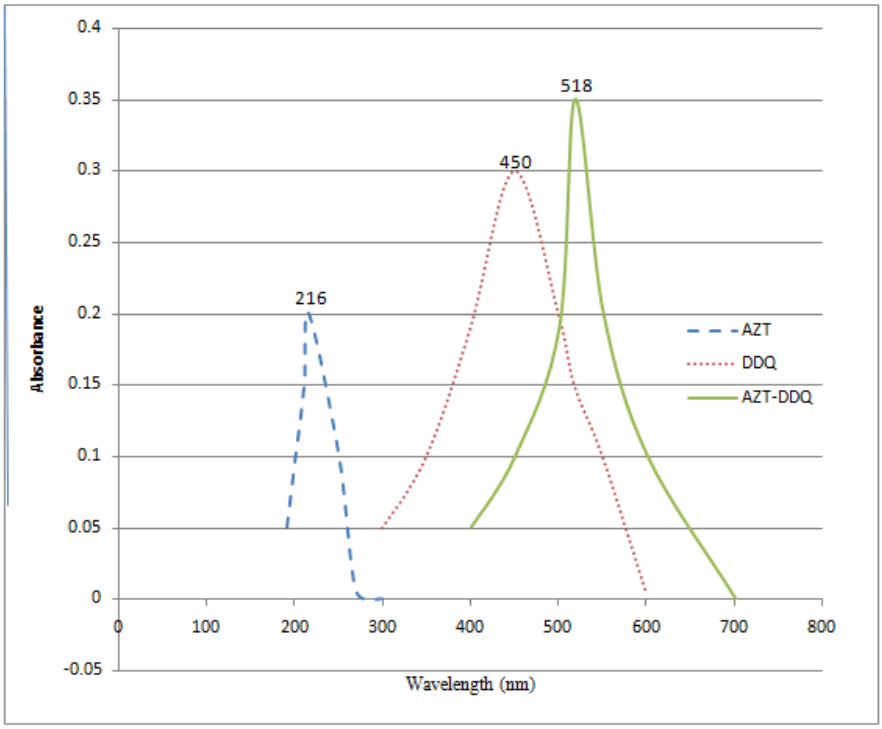

Figure 1A: Graphical Plot of Absorbance versus Wavelengths for Azithromycin, DDQ and Azithromycin-DDQ

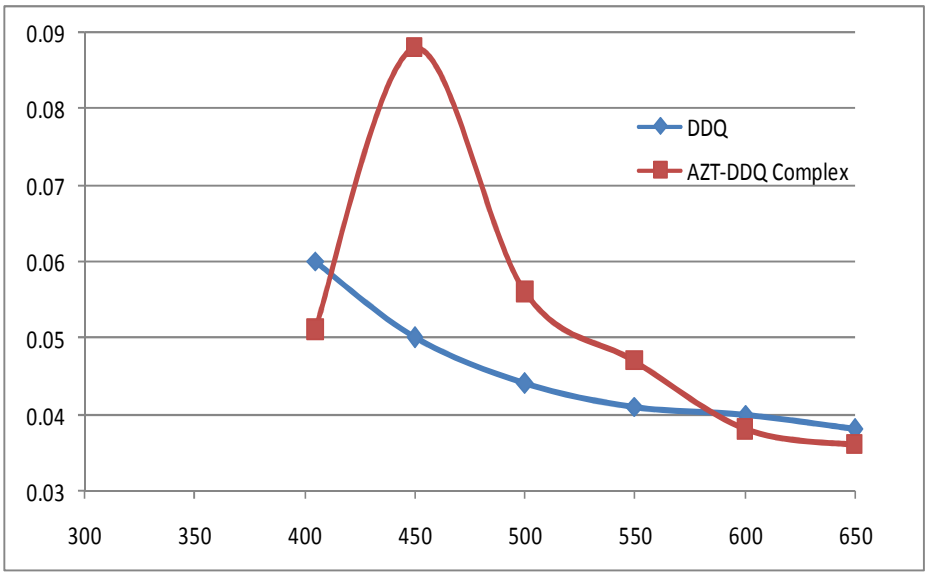

Figure 1B: Graphical Plot of Absorbance versus Wavelengths for DDQ and Azithromycin-DDQ Complex in Thermomax Microwell Reader

Effect of Time on the Formation of the AZT-DDQ Complexes

The azithromycin-DDQ complex formation was progressive until 100 minutes, as shown in figure 2 below. From the 100 minutes, the complex became stable for about 50 minutes before a drop in absorbance was noticed. The implication of this finding is that during subsequent complexation, the solution was allowed to stand for 100 minutes. This is to allow

for full development of the complex. This helped to avoid variations in the absorbance readings of the AZT-DDQ complex.

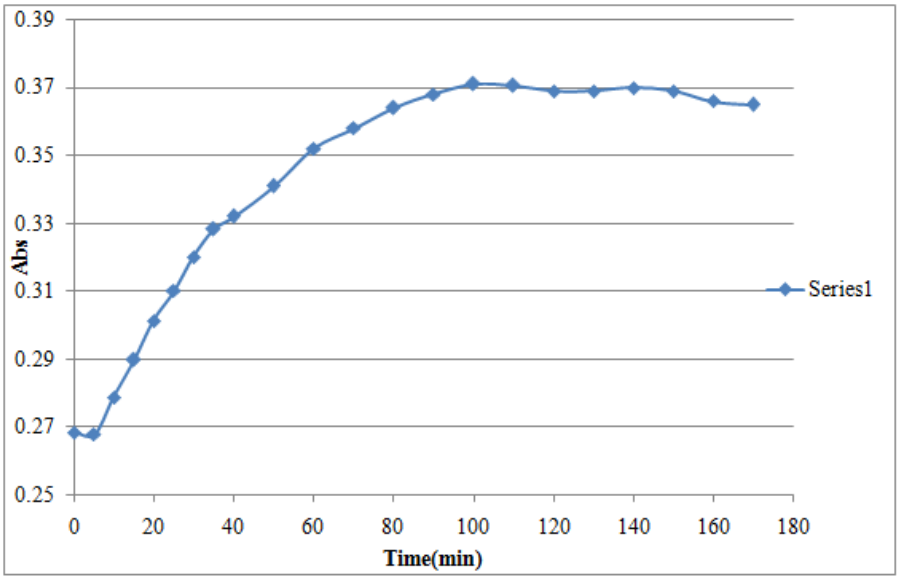

Figure 2: A Graph of Absorbance against Time

Determination of Stoichiometric Ratio of AZT-DDQ Complex

The stoichiometric ratio of the AZT-DDQ complex was determined using Job's method of continuous variation. A 1:2 ratio of charge-transfer complex was indicated for the azithromycin-DDQ interaction (Figures 3). In subsequent experiments, the ratio obtained was used as the basis for the reaction mixtures.

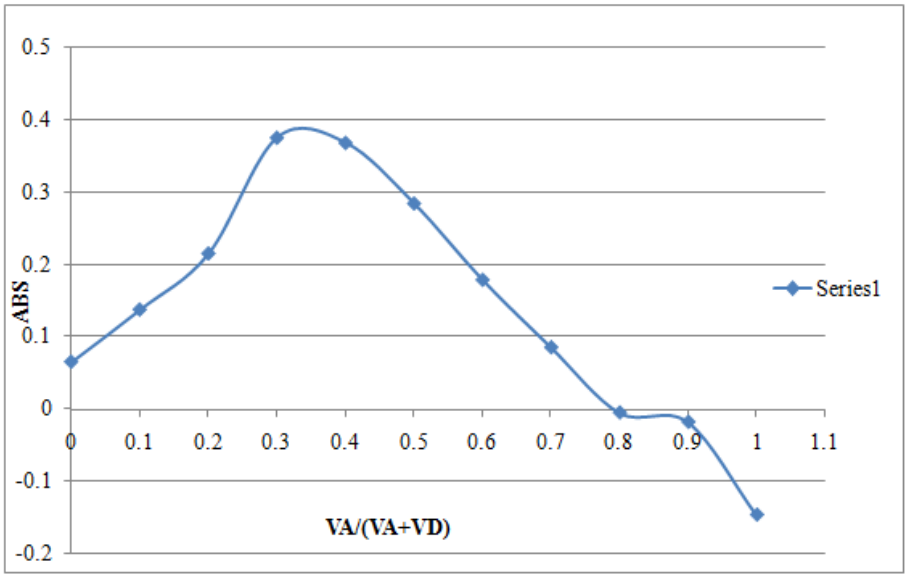

Figure 3: A Graph of Absorbance Against VA/(VA+VD)

Determination of Effect of Ph of AZT-DDQ Complexation

The result of determination of the effect of pH on the AZT-DDQ complex formation showed that the complex was optimally formed between the pH ranges of 4 to 7 as shown in Figure 4. The pH of the unbuffered mixture of AZT and DDQ at the ratio of 1:2 is 4.71, which falls within the range of the optimal complexation pH. This implies that the complex

formation reaction medium need not be buffered.

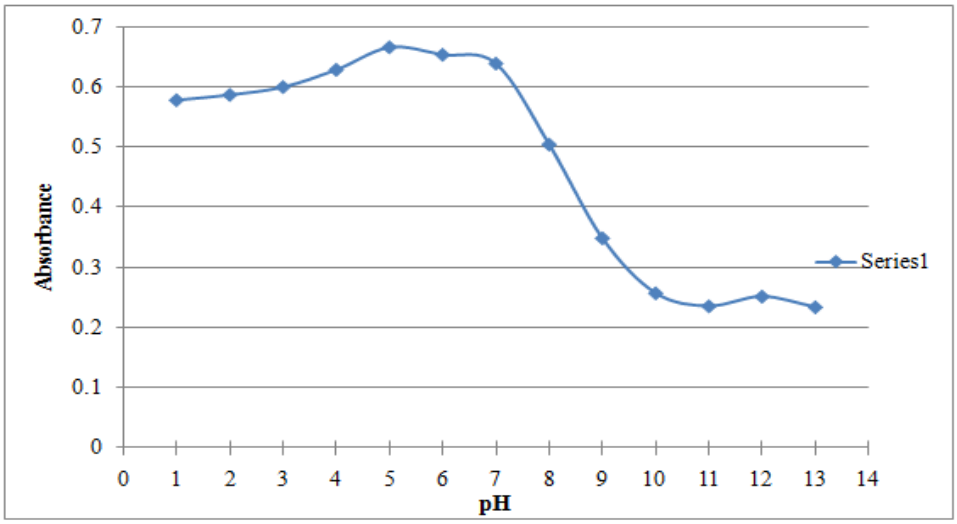

Figure 4: A Graph of Ph against Absorbance

Association Constant, Molar Absorptivity and Thermodynamic Parameters of the Complexes:

The molar absorptivities calculated were almost constant at the different temperatures. This was expected since ideally, it should not vary. Increase in temperature may have led to the dissociation of the formed complexes. The standard enthalpy change, ΔH^o, of the azithromycin-DDQ interaction was obtained from this equation:

$$LogK_c^{DA} = -\frac{\Delta H^\circ}{2.303RT} + constant$$

Gibb's free energy (ΔG^o) and the entropy (ΔS^o) were calculated from the equations below. The results are presented in table 1

$$\Delta G = -RTlnK_c^{(DA)}$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

From the results obtained as shown in table 1**,** the reaction is a spontaneous process. A spontaneous process is the time-evolution of a system in which it releases free energy and moves to a lower, more thermodynamically stable energy state. The sign convention of changes in free energy follows the general convention for thermodynamic measurements, in which a release of free energy from the system corresponds to a negative change in free energy, but a positive change for the surroundings.

Table 1: Association Constant, Molar Absorptivity and Thermodynamic Parameter of the Complex

K	S	Ɛ	LOGK	T(K)	1/T	ΔH^o Kcal/Mol	ΔG^o Kcal/Mol	ΔS^o Kcal/Mol
30.21	23.49	0.042571	1.480151	298	0.0034	28.29	-8429.775	28.3
36.46	19.82	0.050454	1.561817	313	0.0032	29.29	-9342.609	29.94
128.2	69.62	0.014364	2.107888	323	0.0031	40.85	-13011.99	40.41
98.2	51.08	0.019577	1.992111	333	0.003	38.08	-12678.03	38.18

For any spontaneous process, according to the second law of thermodynamic ΔS>=0. If equality holds, then the process is reversible, but such reaction process would take forever to happen. For all real spontaneous processes ΔS> 0, For a spontaneous reaction ΔG = < 0. When ΔS and ΔH are positive, the reaction is spontaneous. From the conditions stated, the process of complex formation between Azithromycin is a spontaneous process.

Result of Determination of Complexation Mode between Azithromycin and DDQ

Molecular simulated docking of Azithromycin and DDQ confirmed the 1:2 ratios obtained from the Job's method of continuous variation. The docking also showed that only one nitrogen atom on the Azithromycin molecule is involved in the complexation mechanism as shown below. The figure 5 below shows the two DDQ molecules round a nitrogen atom of the Azithromycin molecule, with 4.7 Å and 4.8 Å.

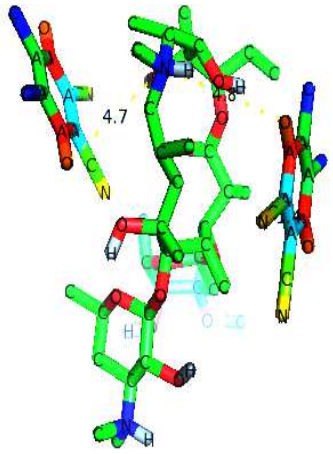

Figure 5: Complexation Mode between Azithromycin and DDQ

Beer's Law Limit

The result of the Beers' limit as shown in figure 6 below shows that the azithromycin-DDQ complex has a linear curve over certain range of concentration, after which the linearity between concentration and absorbance was lost. The graph plateaus from this point.

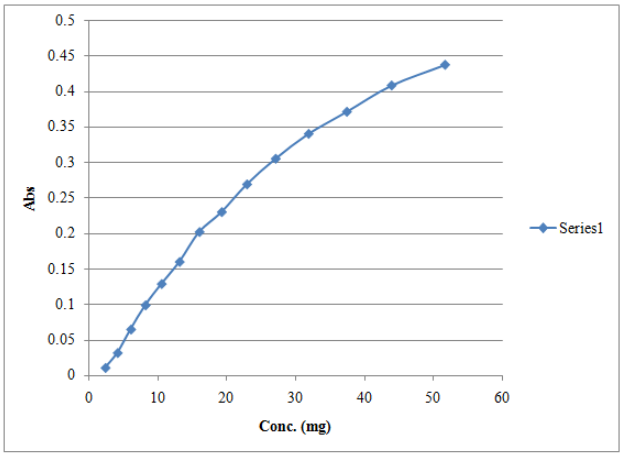

Figure 6: A Graph of Absorbance against Concentration

Assay of Azithromycin Brands

A standard calibration plot for Azithromycin-DDQ complex was constructed by plotting absorbance versus concentration of the drug in mg/ml of the Azithromycin standard solution. A straight line was obtained for the complexed drug as shown in Figure 7 below, indicating that colorimetric analysis with the Thermomax microwell reader can be used for quantitative analysis of the drug in electron donor-acceptor complex formation. The calibration obtained was used in the assay of azithromycin brands.

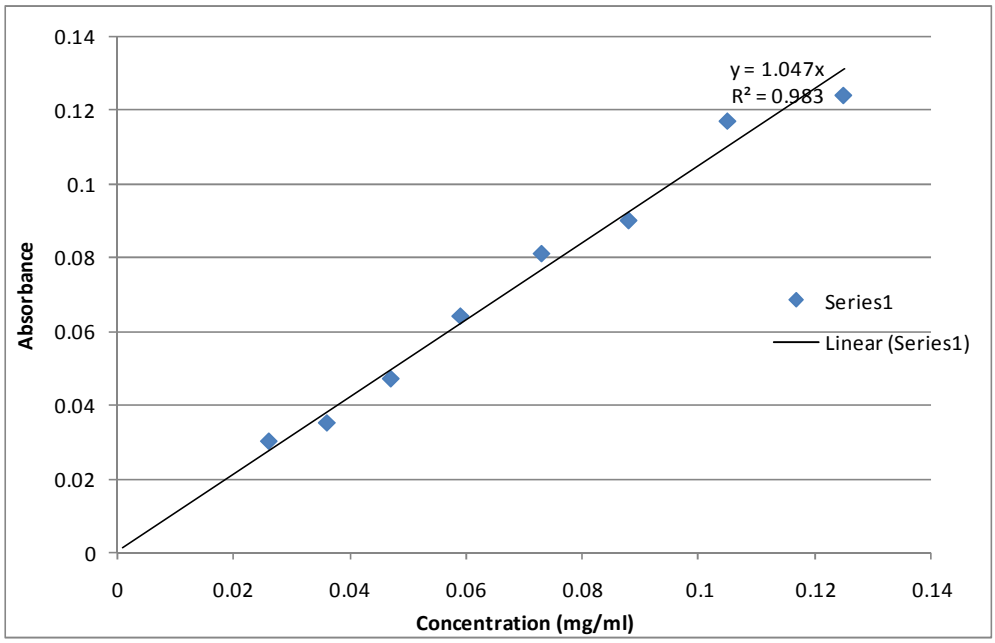

Figure 7: A Graph of Absorbance against Concentration

Table 2: Result of Weight Uniformity and Assay of Azithromycin

S/N	Brand Code	Dosage Form	Country of Origin	Nafdac Reg. No	Batch No	Average Weight(gm)	Absolute Drug Content (%)
1	AZI1	Tablet	India	A4-4136	2933	0.920538	100.05
2	AZI2	Tablet	India	A4-0558	007	0.357426	104.35
3	AZI3	Tablet	India	A4-4348	AZF 01	1.128260	101.13
4	AZI4	Capsule	India	A4-1075	202	0.383586	96.29
5	AZI5	Capsule	India	None	X20096	0.484290	95.21

Validation

A linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to concentration. The linearity of response for the present method was determined by analyzing standard solution of azithromycin in the concentration range of 0.026 – 0.105 mg/ml.

The results showed responses are linear within the concentration range of the analysis. A correlation coefficient of one would indicate a perfectly linear relationship between the concentration of Azithromycin in methanol and absorbance.

The limit of detection (LOD) and limit of quantification (LOQ) were evaluated based on Azithromycin-DDQ standard solution. Azithromycin-DDQ standard solutions were prepared and analyzed for linearity. The obtained results showed that the LOD = 0.023 mg/ml and LOQ = 0.069 mg/ml, using the formula 3.3 x SD/slope, for the Limit of

Detection and 10 x SD/slope, for the Limit of Quantitation. Where SD is the standard deviation of Absorbance reading and the slope is the mean slope of three plots.

The results obtained from intermediate precision (intra-day and inter-day) also indicated a good method of precision.

CONCLUSIONS

The method developed and validated in the present work proved to be an excellent alternative for the azithromycin determination in pharmaceutical formulations. It gave adequate sensitivity and selectivity, allowing the determination of analyte at levels under those found in the sample. The present study described the investigation of the CT complexation parameters, investigation thermodynamic parameters of the CT and the development and validation of a novel microwell-based spectrophotometric assay for the determination of Azithromycin based on its CT reaction with DDQ. In this assay the CT reaction was done and the absorbance reading taken in a UV spectrophotometer. The assay described herein offered the following advantages:

- Reduction in the consumption of organic solvents in the CT- based spectrophotometric analysis, accordingly reduction in the exposures of the analysts to the toxic effects of organic solvents
- Reduction in the analysis cost
- Although the proposed assay was developed and validated for Azithromycin, however it is also anticipated that the same methodology could be used for essentially any analyte that can exhibit CT reaction.

These characteristics make the method very suitable for routine analysis in quality control laboratories in developing countries.

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